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Modulation of osteogenic potential by recombinant human bone morphogenic protein-2 in human periodontal ligament cells: effect of serum, culture medium, and osteoinductive medium

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Hou L-T, Li T-I, Liu C-M, Liu B-Y, Liu C-L, Mi H-W. Modulation of osteogenic potential by recombinant human bone morphogenic protein-2 in human periodontal ligament cells: effect of serum, culture medium, and osteoinductive medium. J Periodont Res 2007; 42: 244–252. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard

Background and Objective: Bone morphogenic proteins are known, in animal models, to promote many developmental processes, including osteogenesis. Clinical trials are currently underway to evaluate the potential of bone morphogenic proteins to promote bone and periodontal regeneration in humans. The aim of this study was to establish an optimal cell culture condition for using to study the biological effects of recombinant human bone morphogenic protein-2 on periodontal ligament cells.

Material and Methods: The roles of serum concentration, types of culture medium (α-modified essential medium or Dulbecco's modified Eagle's medium), the presence of osteoinductive medium (including dexamethasone, ascorbic acid and β-glycerophosphate), and timing of addition of the osteoinductive medium and recombinant human bone morphogenic protein-2, on the expression of alkaline phosphatase were investigated in cultured periodontal ligament cells. Cytochemical stainings and biological assay of alkaline phosphatase were also demonstrated.

Results: Our results suggested that an increased concentration of serum might mask the effect of recombinant human bone morphogenic protein-2 on the expression of alkaline phosphatase in periodontal ligament cells. α-Modified essential medium was found to induce a stronger cytochemical staining of the alkaline phosphatase than Dulbecco's modified Eagle's medium under similar culture conditions. Pre-incubation of cells with osteoinductive medium before the addition of various concentrations of recombinant human bone morphogenic protein-2 enhanced greater alkaline phosphatase expression than the simultaneous presence of both osteoinductive medium and recombinant human bone morphogenic protein-2.

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Conclusion: The findings of this study suggest that the effect of recombinant human bone morphogenic protein-2 on periodontal ligament cells could be efficiently investigated after the proper selection of culture variables and temporal sequence of adding bioactive factors. The optimal culture condition identified in this study might be useful in further studies to elucidate the regulatory mechanism of periodontal ligament cells in periodontal regeneration after stimulation with recombinant human bone morphogenic protein-2.

Periodontal disease is characterized by inflammation and the subsequent loss and/or damage of tooth-supporting tissues, including cementum, bone, and periodontal ligament (1). New formation or regeneration of the disease-deteriorated tissues is a critical issue of clinical periodontology and is still a hot topic of intensive study as a result of the unpredictable outcome of different therapies. Progenitor cells for tissue regeneration have been suggested to derive mainly from periodontal ligament tissues (2–4). The periodontal ligament cell population has many osteoblast-like properties, including the capacity to respond to hormones (vitamin D3, parathyroid hormone) (5) and bone-inductive factors (6,7), to form mineralized nodules *in vitro*, and to express many bone-associated proteins, such as alkaline phosphatase and osteocalcin. Periodontal ligament cells seem to possess some osteogenic and fibrogenic progenitor cells that maintain homoeostasis and aid the regeneration of periodontal tissues (8–10).

Bone morphogenic proteins belonging to the transforming growth factor-β superfamily have long been known as potential inducers of bone formation (11,12) and also as factors involved in regulating the morphogenesis and differentiation of many organ systems (13,14) and tooth development (15,16). *In vitro*, bone morphogenic protein can regulate the expression of osteogenic proteins, including osteocalcin, bone sialoprotein, osteopontin and alkaline phosphatase. (17,18). Results from animal studies (19–21) and human clinical trials (22,23) indicate that bone morphogenic proteins have the ability to stimulate the regeneration of bone and periodontal tissues, including cementum. Although study results have highlighted the potential clinical application of bone morphogenic proteins for periodontal regeneration, studies using human cells to investigate the underlying mechanism of its healing process have not been conducted in detail. Only a limited number of studies have illustrated the modulatory effects of bone morphogenic proteins on the differentiation of the human osteoblastic lineage, especially periodontal ligament cells. Therefore, it is essential to investigate the effects of bone morphogenic proteins on cells isolated from the human periodontal ligament before considering clinical use of bone morphogenic proteins for periodontal regeneration.

Alkaline phosphatase plays an important role as an enzyme marker in osteoblast differentiation and bone mineralization, both *in vivo and in vitro* (24,25). Recombinant human bone morphogenic protein-2 has been reported as a positive regulator for alkaline phosphatase in many cell types, including MC3T3-E1 (26), human mandible bone cells (27), human bone marrow stromal cells (28), human neonatal calvaria cells (29), and rat and human periodontal fibroblasts (30,31). However, different results of previous *in vitro* studies, using different culture conditions, often make comparisons and interpretations relevant to real tissue difficult. According to my knowledge, this issue has not been applied in periodontal ligament cells. Therefore, the aim of this study was to establish a suitable culture condition for using to study the *in vitro* recombinant human bone morphogenic protein-2-induced differentiation of periodontal ligament cells. In this study, the effects of serum concentration, culture medium, pre-incubation in osteoinductive medium (containing dexamethasone, ascorbic acid and β-glycerophosphate), and dose-dependent response of recombinant human bone morphogenic protein-2-induced alkaline phosphatase expression, were tested.

Material and methods

Cell culture

Human periodontal ligament was obtained from adult healthy premolars extracted for orthodontic reasons. Briefly, periodontal ligament tissue was dissected from the mid-third portion of premolar roots, using a sharp surgical scalpel, and then minced into small pieces and placed onto 35-mm tissue culture dishes. The explants were then covered with sterilized glass coverslips and kept in α-modified essential medium (Sigma, St Louis, MO, USA), which contains 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) at 37°C, in 5% CO_2-humidified air. Cells were allowed to grow out of the explants and reached confluence. Cells were then expanded and stored in liquid nitrogen for future use. Periodontal ligament cells between passages four and seven were used in the present study.

Determination of optimal conditions for assaying the modulation of alkaline phosphatase expression by recombinant human bone morphogenic protein-2

Role of medium type In this preliminary study, different serum concentrations (0.2, 2, 5, or 10%) were used to test the differential effect of recombinant human bone morphogenic protein-2 on alkaline phosphatase expression. Periodontal ligament cells, 2×10^4/well, were initially plated in a 24-well culture dish with α-modified essential medium + 10% fetal bovine serum to reach near confluence. Then, the medium of

each dish, each containing different serum concentrations, was changed and cultured for 3 d. Several culture wells were supplemented with osteoinductive ingredients (osteoinductive medium: 50 μg/ml ascorbic acid, 10 mM sodium β-glycerophosphate and 10^{-7} M dexamethasone) simultaneously when changed to different serum conditions. Cells were further stimulated with recombinant human bone morphogenic protein-2 (0, 100, 300, or 500 ng/ml) for an additional 3 d. Cytochemical alkaline phosphatase staining was performed to determine the influence of recombinant human bone morphogenic protein-2 on alkaline phosphatase expression. At the end of the experiment, cultured periodontal ligament cells were thoroughly washed with 1 × phosphate-buffered saline, fixed with 2% paraformaldehyde (Sigma) dissolved in phosphate-buffered saline, and stained cytochemically using the azo-dye coupling technique (32).

Owing to excessive cytochemical staining of alkaline phosphatase in periodontal ligament cells cultured in α-modified essential medium, irrespective of the presence or absence of recombinant human bone morphogenic protein-2, α-modified essential medium was assumed to mask the effect of recombinant human bone morphogenic protein-2. Therefore, Dulbecco's modified Eagle's medium (Gibco) was used for comparison under the same culture condition. Periodontal ligament cells, 2×10^4/well, were initially plated in a 24-well culture dish containing either α-modified essential medium or Dulbecco's modified Eagle's medium + 10% fetal bovine serum. Alkaline phosphatase staining was performed to determine the effects of different culture medium on the level of alkaline phosphatase expression.

Effect of serum concentration To test the effect of serum concentration and osteoinductive medium on the alkaline phosphatase expression of periodontal ligament cells cultured in Dulbecco's modified Eagle's medium, periodontal ligament cells, 2×10^4/well, were initially incubated to confluence in a 24-well culture dish containing regular Dulbecco's modified Eagle's medium + 10% fetal bovine serum. The medium was then replaced with the same medium containing either serum-free medium + 0.2% bovine serum albumin (Sigma) or different concentrations of serum (0.2, 2, or 10%). The cultures were performed in either the presence or absence of osteoinductive medium for 3 d. Alkaline phosphatase staining was performed to determine the differential effect of the serum on alkaline phosphatase expression.

Effect of timing of osteoinductive medium pre-incubation Osteoinductive medium is essential for studying *in vitro* mineralization. Our preliminary study indicated that recombinant human bone morphogenic protein-2 alone did not sufficiently stimulate an alkaline phosphatase response. In order to time the addition of osteoinductive medium to obtain the optimal effect of recombinant human bone morphogenic protein-2, cell cultures were pre-incubated with osteoinductive medium in standard Dulbecco's modified Eagle's medium. Periodontal ligament cells, 2×10^4/well, were initially plated, to reach near confluence, in a 24-well culture dish in Dulbecco's modified Eagle's medium + 10% fetal bovine serum. In one study group, the medium was changed to serum-free Dulbecco's modified Eagle's medium (0.2% bovine serum albumin) containing osteoinductive medium and various concentrations of recombinant human bone morphogenic protein-2 (0, 50, 100 or 300 ng/ml) for 3 d. The other group of confluent cells were pre-incubated in the same serum-free Dulbecco's modified Eagle's medium supplemented with osteoinductive medium for 3 d, and then further stimulated with recombinant human bone morphogenic protein-2 (0, 50, 100, or 300 ng/ml) for an additional 3 d in the same medium. Alkaline phosphatase staining was performed to determine the effect of pre-incubation in osteoinductive medium for recombinant human bone morphogenic protein-2 stimulation of alkaline phosphatase expression.

Dose–response effect of recombinant human bone morphogenic protein-2 on alkaline phosphatase expression

Periodontal ligament cells were prepared and cultured, as mentioned above. After the cells reached confluence, the culture medium was replaced with either serum-free (0.2% bovine serum albumin) Dulbecco's modified Eagle's medium, or serum-free (0.2% bovine serum albumin) Dulbecco's modified Eagle's medium supplemented with osteoinductive medium, and maintained for 3 d. Cultured cells were further stimulated with recombinant human bone morphogenic protein-2 (0, 50, 100, or 300 ng/ml) for an additional 3 d in the same culture medium. At the end of the experiment, cells were scraped off from the dish into 1 ml of Tris-HCl buffer and then sonicated before measurement of cellular DNA and alkaline phosphatase activity.

To assay alkaline phosphatase activity, the harvested cells were sonicated in 1 ml of Tris-HCl buffer (10 mM, pH 7.4). The sonicates were used as an enzyme solution to determine alkaline phosphatase activity, as measured by the release of *p*-nitrophenyl phosphate, and the color reaction product was measured at 410 nm wavelength on a Beckman DU-65 spectrophotometer (Beckman, Fullerton, CA, USA) (32).

The cellular sonicates were also used to measure cellular DNA content. After calibrating the spectrophotometer on the cuvette containing 1 ml of TE buffer (10 mM Tris-HCl/1 mM EDTA), the absorbances of the experimental samples, containing 10 μl of cellular sonicates and 990 μl of TE buffer, were measured at 260 nm on a Beckman DU-65 spectrophotometer (Beckman) (32).

Statistical analysis

Measurements of alkaline phosphatase activity were expressed as the mean activity (± standard deviation) of duplicate culture wells. Each sample harvested from cultures stimulated in the presence or absence of recombinant human bone morphogenic protein-2

was compared. Significant analysis was tested using two-way analysis of variance, and intergroup differences were determined by Fisher's pairs least significant difference (PLSD) test. A p-value of 0.05 or 0.01 was considered to be statistically significant.

Results

When periodontal ligament cells were cultured in α-modified essential medium containing different concentrations of serum and in either the presence (Fig. 1, lanes b, d, and f) or absence (Fig. 1, lanes a, c, and e) of osteoinductive medium, alkaline phosphatase expression was affected by the concentration of serum, irrespective of the dose of recombinant human bone morphogenic protein-2. Similar results were also found in α-modified essential medium containing 10% serum (data not shown). Cells cultured in the presence of 0.2% fetal bovine serum had the lowest alkaline phosphatase expression (Fig. 1, lanes a and b). In the presence of osteoinductive medium, recombinant human bone morphogenic protein-2-stimulated alkaline phosphatase expression was only observed in medium containing 0.2% fetal bovine serum (Fig. 1, lane b). However, no dose–response effect of recombinant human bone morphogenic protein-2 was found in α-modified essential medium.

When comparing alkaline phosphatase expression of confluent periodontal ligament cells in α-modified essential medium (Fig. 2A,C) and Dulbecco's modified Eagle's medium (Fig. 2B,D), in the presence of 10% fetal bovine serum, cells cultured in α-modified essential medium showed significantly higher alkaline phosphatase expression than those cultured in Dulbecco's modified Eagle's medium. Fetal bovine serum is known to contain multiple growth factors and some differentiation factors, which might stimulate alkaline phosphatase expression in α-modified essential medium. In order to investigate factors that modulate the recombinant human bone morphogenic protein-2-stimulatory effect on the alkaline phosphatase expression of cells cultured in Dulbecco's modified Eagle's medium, the effect of serum and osteoinductive medium pre-incubation were examined (Fig. 3). Four serum concentrations (0.2%, 2%, 10% and serum-free + 0.2% bovine serum albumin) were investigated. Similarly to the cells cultured in α-modified essential medium, alkaline phosphatase expression was increased at higher serum concentrations in cells cultured in Dulbecco's modified Eagle's medium (Fig. 3A,B). Cells cultured in Dulbecco's modified Eagle's medium pretreated with osteoinductive medium revealed a higher level of alkaline phosphatase activity (Fig. 3B, e, f, g and h) than those not pretreated.

In order to avoid an unwanted influence of serum on recombinant human bone morphogenic protein-2, serum-free Dulbecco's modified Eagle's medium supplemented with 0.2% bovine serum albumin was used in the latter study to investigate the short-term effect of recombinant human bone morphogenic protein-2 on alkaline phosphatase expression. Serum-free medium enables investigation of the role of cellular responses to one specific factor and thus can remove the influence of numerous reagents presented in serum.

In the present study, confluent periodontal ligament cells were pre-incubated with osteoinductive medium in serum-free Dulbecco's modified Eagle's medium (0.2% bovine serum albumin) for 3 d and then stimulated with recombinant human bone morphogenic protein-2 for an additional 3 d. Recombinant human bone morphogenic protein-2 was found to exhibit a dose-dependent increase of alkaline phosphatase staining (Fig. 4B). In the absence of pre-incubation with osteoinductive medium, no obvious increase of alkaline phosphatase activity was

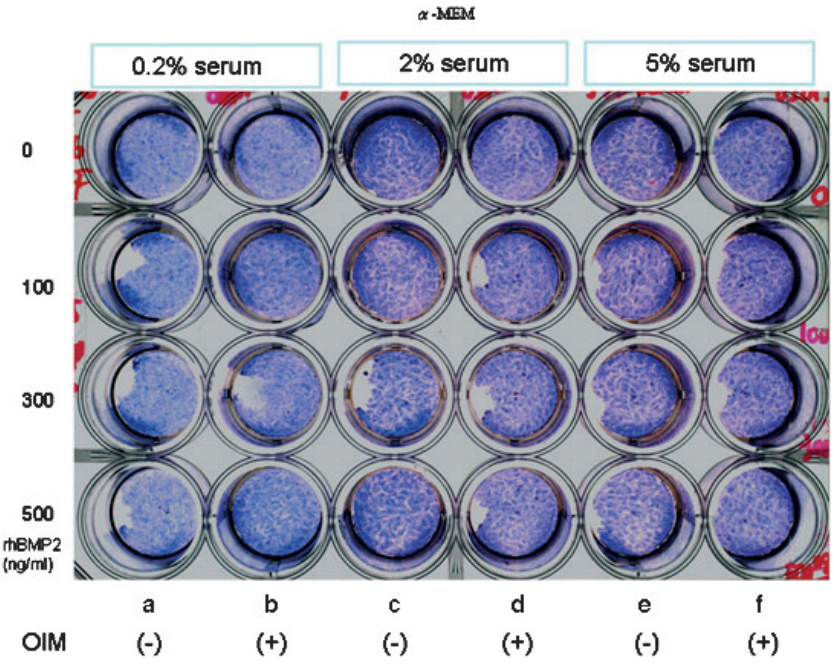

Fig. 1. Effect of serum concentration on recombinant human bone morphogenic protein-2-stimulated alkaline phosphatase expression of periodontal ligament cells in α-modified essential medium. Periodontal ligament cells (2×10^4/well) were initially cultured to confluence in α-modified essential medium, containing 10% fetal bovine serum, in 24-well culture dishes. Then, the medium was replaced with that containing different concentrations of serum and incubation was continued for 3 d, as shown. Some wells (b, d and f) were additionally supplemented with osteoinductive medium simultaneously during the change of medium. Recombinant human bone morphogenic protein-2 was added at the indicated concentration for an additional 3 d. DMEM, Dulbecco's modified Eagle's medium; OIM, osteoinductive medium; rhBMP2, recombinant human bone morphogenic protein-2.

apparent (Fig. 4A). Similar results were also found in a study where cells were pre-incubated with osteoinductive medium in Dulbecco's modified Eagle's medium containing 2% serum (data not shown).

Cell culture assay conditions included serum-free Dulbecco's modified Eagle's medium (0.2% bovine serum albumin) and pre-incubation with osteoinductive medium. When cells were cultured in Dulbecco's modified Eagle's medium without pre-incubation with osteoinductive medium, recombinant human bone morphogenic protein-2 (0, 50, 100, or 300 ng/ml) appeared to have only mild effects on alkaline phosphatase expression (Table 1 and Fig. 5). The change was not statistically significant. When pre-incubated with osteoinductive medium, recombinant human bone morphogenic protein-2 (50, 100 or 300 ng/ml) showed a significantly enhanced dose–response increase of alkaline phosphatase activity (Table 1 and Fig. 5). In comparison with cells pre-incubated in osteoinductive medium and standard Dulbecco's modified Eagle's medium, supplementation with osteoinductive medium significantly increased the alkaline phosphatase activity, irrespective of the concentration of recombinant human bone morphogenic protein-2 (Table 1).

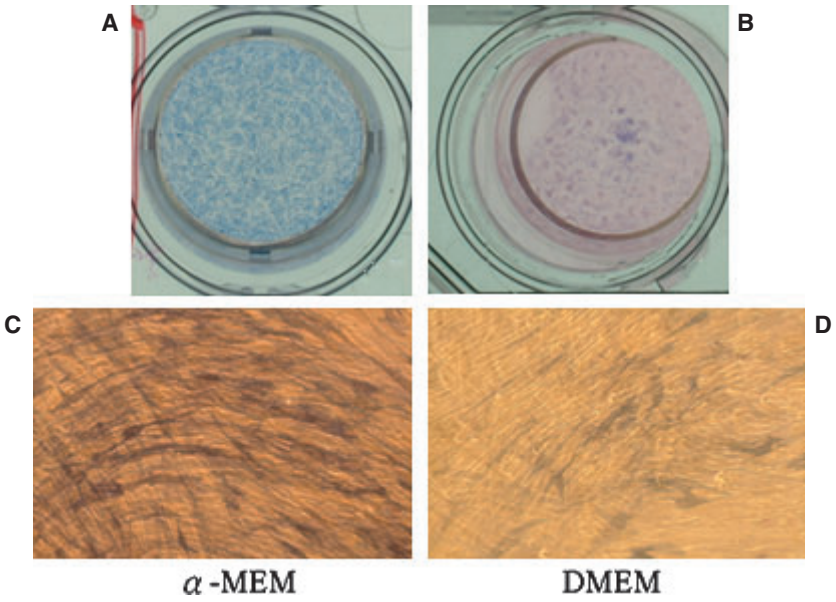

Fig. 2. Alkaline phosphatase staining of confluent periodontal ligament cells cultured in either α-modified essential medium (a, c) or Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (b, d). Cultured periodontal ligament cells in the α-modified essential medium showed a significantly higher intensity of alkaline phosphatase staining than those cultured in Dulbecco's modified Eagle's medium. (a, b: culture dish; c, d: 100× magnification). α-MEM, α-modified essential medium; DMEM, Dulbecco's modified Eagle's medium.

Discussion

Serum is frequently added to the defined basal medium as a source of certain nutritional and macromolecular growth factors essential for cell growth (33). There are over 1000 different components in serum, and the best supplementation to a basal medium is fetal bovine serum, which is frequently used for all types of cell culture. Serum also supplies attachment and growth factors – elements for cell proliferation and differentiation (33).

However, the disadvantages of serum include compositional variability, the stimulation or inhibition of cell growth, and unwanted induction of differentiation (33). In our studies, serum strongly stimulated dose-dependent alkaline phosphatase expression, either in α-modified essential medium or Dulbecco's modified Eagle's medium, which seemed to over-ride the effect of recombinant human bone morphogenic protein-2. This fact was confirmed by the finding that a dose-dependent response to recombinant human bone morphogenic protein-2 was detected by using serum-free or low-serum conditions. The latter strategy seems to be popular in *in vitro* studies investigating unique cellular responses to a single specific factor, which may eliminate the unwanted influence of numerous components present in serum. For short-term analysis of alkaline phosphatase expression, cells cultured in serum-free medium supplemented with bovine serum albumin have been used in many studies (30,34,35). In a study of four human periodontal ligament cell lines, Kobayashi *et al.* demonstrated that under serum-free conditions (with 0.5% bovine serum albumin), the addition of recombinant human bone morphogenic protein-2 at concentrations of > 50 ng/ml significantly stimulated alkaline phosphatase activity, in addition to causing an enhancement of 3,5-cyclic adenosine monophosphate accumulation (30).

Both α-modified essential medium and Dulbecco's modified Eagle's medium, used in the present study, were suitable for supporting the growth of different types of cells. However, there are some differences in their composition, and this could potentially affect the behavior of the cells *in vitro* (36). The alkaline phosphatase expression of periodontal ligament cells may behave like the bone marrow-derived cells in some aspects. Our results showed that periodontal ligament cells cultured in Dulbecco's modified Eagle's medium had a lower alkaline phosphatase activity than periodontal ligament cells cultured in α-modified essential medium under the same experimental conditions. This might be a result of the fact that Dulbecco's modified Eagle's medium favors the maintenance of progenitor cells and that α-modified essential medium could promote initial recruitment into the osteogenic lineage. It may also be a result of the fact that α-modified essential medium contains 50 μg/ml of ascorbic acid in its basal components and thus promotes osteoblast differentiation (37). Whether or not the

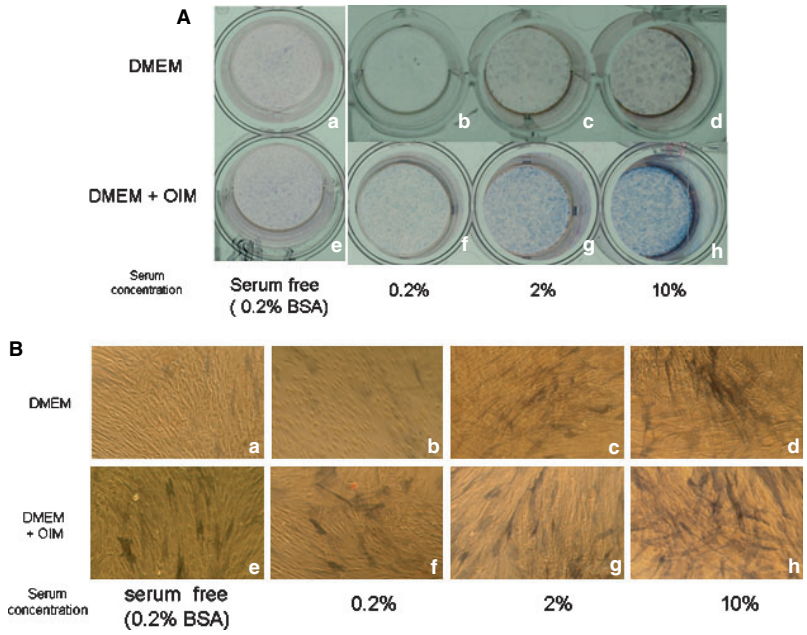

Fig. 3. Effect of incubation with osteoinductive medium and of serum concentration on the alkaline phosphatase expression of periodontal ligament cells cultured in Dulbecco's modified Eagle's medium. (A, culture wells; B, 100× magnification). Periodontal ligament cells (2×10^4/well) were initially cultured to confluence in Dulbecco's modified Eagle's medium, containing 10% fetal bovine serum, in a 24-well culture dish. The culture medium was then changed to Dulbecco's modified Eagle's medium (wells a, b, c and d), which contained a different concentration of serum, for 3 d (upper row in A and B). The culture wells shown in e, f, g and h contained osteoinductive medium (lower row in A and B). Magnified pictures taken from each well in (A) are shown in (B). α-MEM, α-modified essential medium; OIM, osteoinductive medium; recombinant human bone morphogenic protein-2, recombinant human bone morphogenic protein-2.

phenotype of progenitor cells in periodontal ligament cell populations, which were cultured in Dulbecco's modified Eagle's medium, could be easily maintained still needs future study.

In the study of Jaiswal *et al.*, human bone marrow-derived cells were cultured in four types of media (Dulbecco's modified Eagle's medium, α-modified essential medium, Dulbecco's modified Eagle's medium/F-12 and BGJ_b) in the presence or absence of osteoinductive medium (36). They found that cells cultured in different media exhibited a different intensity of alkaline phosphatase staining. In their study, alkaline phosphatase staining of cells cultured in α-modified essential medium was higher than that of cells cultured in Dulbecco's modified Eagle's medium. Cells cultured in Dulbecco's modified Eagle's medium showed the lowest basal alkaline phosphatase activity compared with cells cultured in any other medium tested. However, the addition of osteoinductive medium to Dulbecco's modified Eagle's medium increased the alkaline phosphatase activity by ≈13-, 11-, and 6-fold on days 8, 12, and 16, respectively, while the addition of osteoinductive medium to α-modified essential medium increased alkaline phosphatase activity by only 4-, 4-, and 2-fold, for the same culture periods (36). Different types of culture medium therefore seem to have a different effect on osteogenic activity.

In our studies, pre-incubation with osteoinductive medium was found to enhance the stimulatory effect of recombinant human bone morphogenic protein-2 on alkaline phosphatase expression. Meanwhile, recombinant human bone morphogenic protein-2 exhibited a dose-dependent stimulation of alkaline phosphatase expression after pre-incubation in the osteoinductive medium. Similarly, recombinant human bone morphogenic protein-2 has been reported to enhance the alkaline phosphatase expression in murine periodontal ligament cells after pre-incubation in osteoinductive medium (31). In this study, Saito and colleaques reported that an SV40 large-T antigen gene-transfected periodontal ligament cell line simulated the gene expression of both the periodontal ligament tissues *in vivo* and mass culture cells *in vitro* (31). This cell line expresses genes such as alkaline phosphatase, type I collagen, periostin and runt-related transcription factor 2, but does not have gene expression for bone sialoprotein and osteocalcin in normal medium. With the addition of recombinant human bone morphogenic protein-2, alkaline phosphatase expression increased and mineralized nodules were formed in the differentiation medium (its constituents were similar to those of our osteoinductive medium). In addition, the gene expression of bone sialoprotein, osteocalcin and runt-related transcription factor 2/core binding factor 1/osteoblast-specific factor-2 was markedly enhanced in culture conditions with pre-inculation of differentiation medium and the addition of recombinant human bone morphogenic protein-2. These data, together with those of the present study, support the notion that the periodontal ligament cells are closely related to the osteogenic cell lineage or postnatal stem cells in this tissue compartment, as suggested by many studies (5–10). The osteoinductive medium or differentiation medium containing 50 μg/ml of ascorbic acid, 10 mM β-glycerophosphate and 10^{-7} M dexamethasone seems to be responsible for commitment of periodontal ligament cells to the effect of recombinant human bone morphogenic protein-2 in the present study. These factors are known to be essential for studying *in vitro* mineralization. The stimulatory effect may be relevant to three constituents of osteoinductive medium which will be explained later.

Dexamethasone is known to promote expression of the differentiated phenotype in cells of the osteoblast lineage, and appears able to accelerate the proliferation and survival of pri-

mitive cells, expressing STRO-1 antigen, from bone-derived culture (10). STRO-1-positive cells were thought to be precursors of the osteoblast phenotype. Periodontal ligament cells were recently reported to contain STRO-1-positive cells (10). These data suggest that the addition of dexamethasone, in the present study, might permit progress of progenitor cells in the periodontal ligament pool towards osteoblast differentiation.

In addition, most human femur bone marrow stromal cells appeared to be incapable of expressing elevated alkaline phosphatase levels in direct response to bone morphogenic proteins. Pre-treatment with dexamethasone in the early passage of stromal cells would enhance recombinant human bone morphogenic protein-2-stimulated alkaline phosphatase expression in most cultured bone marrow-derived cells (17). Recombinant human bone morphogenic protein-2 enhanced alkaline phosphatase and runt-related transcription factor 2/core binding factor 1/osteoblast-specific factor-2 expression in the cultured medium containing dexamethasone and this has also been reported in other studies (31,34). Therefore, the presence of dexamethasone may directly or indirectly favor the recombinant human bone morphogenic protein-2 effects on alkaline phosphatase and expression of other bone-related protein in periodontal ligament cells.

Ascorbic acid is known to function as a cofactor in the hydroxylation of lysine and proline residues in collagen, and is essential for the normal synthesis and secretion of collagen. In murine dental follicle cells and cementoblasts (38), recombinant human bone morphogenic protein-2 was reported to enhance osteocalcin mRNA expression in the presence of ascorbic acid. Recombinant human bone morphogenic protein-2 produced only a small stimulation of alkaline phosphatase activity in fetal rat calvarial osteoblasts grown in the absence of ascorbic acid, whereas the effect was greatly enhanced in the presence of ascorbic acid (39).

Reports show that the interaction of integrin and collagen are critical at steps upstream of the transcriptional or post-transcriptional regulation of mRNA for alkaline phosphatase expression (40). In one study, bone morphogenic protein-2 receptors were found to colocalize/overlap with αβ integrins, and the intact function of αβ integrins was found to be essential in bone morphogenic protein-2 activity (41). Ascorbic acid was also demonstrated to induce expression of α2β1 integrins in periodontal ligament cells (37). These data indicate that, in the present study, the effects of recombinant human bone morphogenic protein-2 on alkaline phosphatase expression of periodontal ligament cells may be facilitated by interactions of type I collagen and integrins that were induced by ascorbic acid. This hypothesis needs to be further investigated.

β-Glycerophosphate has been used routinely by researchers to study different functional aspects of bone cells and *in vitro* mineralization. β-Glycerophosphate was reported to provide a source of phosphate ion when hydrolyzed by bone cells, and this locally increased phosphate ion helps in deposition of the mineral matrix (42). In the present study, no separate research on the role of β-glycerophosphate was conducted, and its role in

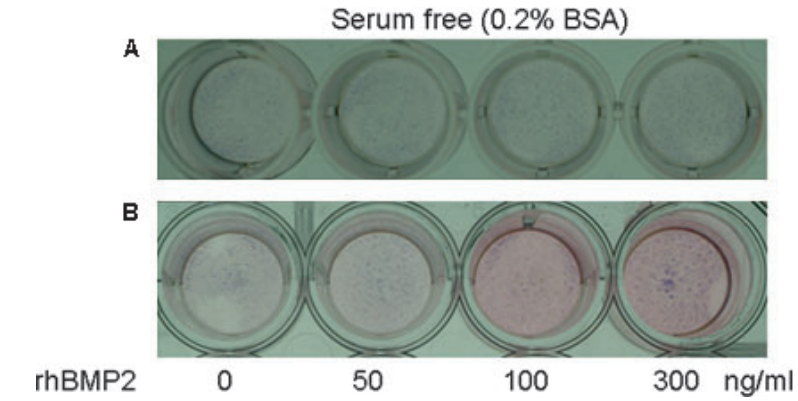

Fig. 4. Effect of pre-incubation with osteoinductive medium on recombinant human bone morphogenic protein-2 stimulation of alkaline phosphatase expression in periodontal ligament cells. Periodontal ligament cells (2×10^4/well) were initially cultured to confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in 24-well culture dish. (A) Cultures were incubated in serum-free (0.2% bovine serum albumin) Dulbecco's modified Eagle's medium + osteoinductive medium + recombinant human bone morphogenic protein-2 for 3 d after the cells reached confluence. (B) The same culture medium as in (A) was used for 3 d after pre-incubation in the same Dulbecco's modified Eagle's medium + osteoinductive medium for an additional 3 d. rhBMP2, recombinant human bone morphogenic protein-2.

Table 1. Dose–response effect of recombinant human bone morphogenic protein-2 (rhBMP2) on alkaline phosphatase (ALPase) activity in cultured periodontal cells pre-incubated with osteoinductive medium (OIM)

		ALPase activity				
rhBMP2 (ng/mL)	N	DMEM		OIM		
0	2	0.0434 ± 0.0026	*	0.0570 ± 0.0032	**	*
50	2	0.0420 ± 0.0045	**	0.0686 ± 0.0040	**	*
100	2	0.0459 ± 0.0041	**	0.0704 ± 0.0029		
300	2	0.0524 ± 0.0020	**	0.0877 ± 0.0034		
		NS				

All data were evaluated in duplicate cultures and the results are expressed as means ± SD. Comparisons of statistical significance for ALPase activity among and between experimental and control.
*, $p < 0.05$.
**, $p < 0.01$.
NS, not significant.

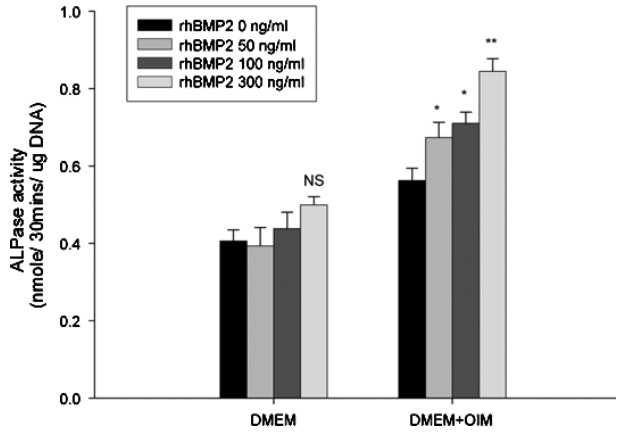

Fig. 5. Dose-dependent response of recombinant human bone morphogenic protein-2 on the stimulation of alkaline phosphatase activity. Periodontal ligament cells (2×10^4/well) were initially cultured to confluence in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum in a 24-well culture dish. Then, the medium was replaced with serum-free Dulbecco's modified Eagle's medium (0.2% bovine serum albumin), with or without the presence of osteoinductive medium, for 3 d. Recombinant human bone morphogenic protein-2 (0, 50, 100, and 300 ng/ml) was added and culture continued for an additional 3 d. All data were evaluated in duplicate cultures and the results were expressed as means ± standard deviation. Statistical significance was determined by analysis of variance. (*$p < 0.05$, **$p < 0.01$; NS, not significant). BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; OIM, osteoinductive medium; rhBMP2, recombinant human bone morphogenic protein-2.

the expression of alkaline phosphatase needs further examination.

In conclusion, our data demonstrated that modulation of alkaline phosphatase expression by adding recombinant human bone morphogenic protein-2 to periodontal ligament cells could not be detected in α-modified essential medium containing fetal bovine serum or even after pre-incubation with osteoinductive medium. The presence of serum in both α-modified essential medium and Dulbecco's modified Eagle's medium was found to mask the biological effect of recombinant human bone morphogenic protein-2 on alkaline phosphatase expression. Serum-free conditions and the use of Dulbecco's modified Eagle's medium were demonstrated to be an appropriate choice for using to explore the biological effect of recombinant human bone morphogenic protein-2 in periodontal ligament cells. Evidence from the present study also suggested that pre-incubation with osteoinductive medium using Dulbecco's modified Eagle's medium could provide a microenvironment favoring the effect for the bone morphogenic proteins. The optimal culture condition identified in study will be useful to evaluate regulation of bone marker gene expression in periodontal ligament cells.

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